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(54) **PROCEDE ET KIT POUR IDENTIFIER DES INTERACTIONS ENTRE DES PROTEINES OU DES PEPTIDES**

(54) **METHOD AND KIT FOR IDENTIFYING INTERACTIONS BETWEEN PROTEINS OR PEPTIDES**

(57) L'invention concerne un procédé et un kit pour identifier des interactions entre des protéines ou des peptides au moyen de transfert d'énergie par résonance-fluorescence.

(57) The invention relates to a method and kit for identifying interactions between proteins or peptides by means of fluorescence resonance energy transfer.

ABSTRACT

The present invention relates to a method and to a kit for identifying interactions between proteins or peptides by means of fluorescence-resonance energy transfer.

DESCRIPTION

METHOD AND KIT FOR IDENTIFYING INTERACTIONS BETWEEN PROTEINS OR PEPTIDES

The present invention relates to a method and to a kit for the identification or determination of interactions between proteins or peptides by means of fluorescence-resonance energy transfer.

The identification and analysis of interactions between different proteins or peptides or fragments thereof represents an important problem of biomedical research and biotechnology. At the end of the 1980's a system was consequently developed, which acquired great research significance under the name "Yeast Two-Hybrid-System" (Fields et al., Nature, vol. 340, pp 245-247, 1989). This system is based on the discovery that cellular transcription activators, such as e.g. GAL4 or lexA from yeast could be decomposed in two independent function domains. Both domains are normally a component of a protein in the nucleus of the yeast cell, which binds to specific activating sequences of different target genes and regulates the transcription thereof. One domain, the DNA binding domain (BD), specifically binds to a particular DNA target sequence (upstream activating sequence) in the vicinity of the target gene promoter. The other domain, the activating domain (AD), increases the transcription rate of the target gene by interaction with the transcription initiation complex, which is bound to the promoter of the target gene. In the "Yeast Two-Hybrid System" this structure is utilized by transcription factors in modified form. The DNA binding domain (BD) of GAL4 or lexA is there expressed as a fusion protein with a "baiting protein or peptide" in yeast cells. This fusion protein also has a nucleus location signal through which it is transported into the yeast nucleus. The baiting-fusion protein is bound there to a target sequence (UAS), which in the yeast strain used is located in the vicinity of promoters of two reporter genes (e.g. auxotrophic marker (HIS3) and enzymatic marker (lacZ)). This leads to a constellation in which the baiting protein or peptide is exposed in direct, spatial proximity to the reporter gene promoter. Additionally a second fusion protein is expressed in the same yeast cell. It comprises the activating domain (AD) of GAL4 or lexA and a catching/preying protein or peptide. It also has a nucleus location signal. The catching-fusion protein is consequently also transported into the yeast nucleus. If the catching protein and the baiting protein exposed at the UAS physically interact with

one another, then there is an increase in the statistical probability that the activating domain is located in the vicinity of the reporter gene promoter. This leads to an increase in the transcription of the reporter genes, whose extent is proportional to the strength of the interaction between the baiting and catching protein. The "Yeast Two-Hybrid System" can be used both for the quantitative analysis of known bait/catch pairs and for the identification of unknown catching proteins or peptides. Possible catching proteins are e.g. a cDNA library or a combinatory peptide library.

Despite the above-described multiplicity of uses the "Yeast Two-Hybrid System" suffers from limitations due to the transcription-dependent detection system. They occur e.g. if the catching and/or baiting protein contains localization signals. These interfering localization signals are e.g. hydrophobic transmembrane domains, such as occur in many membrane proteins. They lead to a transportation of the fusion protein into the cellular membrane, whilst the nucleus localization signal of the fusion protein is ignored. Therefore interactions with proteins having such transmembrane domains cannot be detected. A further problem arises on screening for the analysis of cDNA libraries or combinatory peptide libraries, which can only be carried out to a limited extent. Due to their complexity (e.g. more than 10 billion possible variants for a decapeptide) the latter make efficient screening impossible. The analysis of possible interaction partners has therefore hitherto taken place in that a maximum of 50,000 transformed yeast cells per agar plate are plated out and incubated initially for 3 to 7 days in the incubator. This means that a typical test of approximately 5 million possible fusion protein pairs requires the use of 100 agar plates, which must be thoroughly tested. Thus, the "Yeast Two-Hybrid System" involves high material, time, labour and culture space costs for this screening process type. In addition, there is no known automated analysis process, which would reduce and standardize the high laboratory staff costs.

Thus, the problem of the present invention is to provide a method and a corresponding kit for the identification of interactions between proteins or peptides, which at least partly avoid the aforementioned disadvantages and which can in particular be carried out in an automated manner.

They lead to a transportation of the fusion protein into the cellular membrane, whilst the nucleus localization signal of the fusion protein is ignored. Therefore interactions with proteins having such transmembrane domains cannot be detected. A further problem arises on screening for the analysis of cDNA libraries or combinatory peptide libraries, which can only be carried out to a limited extent. Due to their complexity (e.g. more than 10 billion possible variants for a decapeptide) the latter make efficient screening impossible. The analysis of possible interaction partners has therefore hitherto taken place in that a maximum of 50,000 transformed yeast cells per agar plate are plated out and incubated initially for 3 to 7 days in the incubator. This means that a typical test in the order of 5 million possible fusion protein pairs requires the use of 100 agar plates, which must be thoroughly tested. Thus, the "Yeast Two-Hybrid System" involves high material, time, labour and culture space costs for the screening processes. In addition, there is no known automated analysis process, which would reduce and standardize the high laboratory staff costs.

International patent application WO 97/27212 describes a screening method with which peptide sequences are identified which measurably modify the phenotype of a cell. The method is based on the measurement of the phenotype change after expression of the peptide in the cell. Initially no interactions of peptides or proteins are investigated. Only in a second step is it possible to carry out a closer investigation of the identified, active peptide sequences. For this purpose various known methods can be alternatively used, e.g. the absence of normal cellular functions can be measured, a two-hybrid system in mammalian cells can be used or a fluorescence-resonance energy transfer system can be employed.

The literature already describe some test systems for investigating known interaction partners, which utilize fluorescence-resonance energy transfer. For example, in international patent application WO 91/12530 a fluorescence test is described for investigating interactions of specific binding partners, particularly with respect to immunoassays. European patent application EP 0242527 describes an analysis process for interacting analytes using the energy transfer system.

Thus, the problem of the present invention is to provide a method and a corresponding kit for the identification of interactions between proteins or peptides, which at least partly avoid the aforementioned disadvantages and which can in particular be carried out in an automated manner.

This problem is solved by the subject matter of the claims and their wording is hereby made by reference into part of the content of the present description.

Amended sheet

This problem is in particular solved by a method in which at least two proteins or peptides are coupled to different fluorescent components, the absorption and emission spectra of the fluorescent components overlapping in such a way that fluorescence-resonance energy transfer (FRET) is possible and the components are so brought together by an interaction between the proteins or peptides that FRET occurs and is measured. The genetic information for at least two fusion peptides or proteins having a peptide or protein fraction and in each case one different fluorescent protein or peptide is present in an expression system introduced into a host cell, the emission and absorption spectra of the fluorescent proteins or peptides overlapping in such a way that fluoresence-resonance energy transfer (FRET) occurs and is measured in the host cell.

Within the scope of the present invention the terms "protein" and "peptide" are mutually interchangeable and contain an amino acid line-up of any length and complexity, i.e. dipeptides, oligopeptides, polypeptides, complete proteins, fragments thereof, antibodies, domains, epitopes, etc. They can in particular be combinatory peptide libraries or the expression products of cDNA libraries. The proteins/peptides to be investigated for interactions are subject to no restrictions and can be freely chosen.

According to the invention, the term fluorescent component is in particular understood to mean a material or particles having a fluorescent marking at the surface. They can be latex particles or matrices, as are of a conventional nature for automatic protein synthesis.

According to the invention coupling means a more or less firm binding of the

fluorescent component to the protein(s) or peptide(s). This bond tends to be of a more adsorptive nature or can also be a covalent bond. In a preferred embodiment the protein or peptide and the fluorescent component can be present in the form of a fusion protein or peptide. Coupling can also take place by means of a linker. The latter covers connections of all types suitable for linking two molecules.

The method according to the invention is inter alia based on the known phenomenon of fluorescence-resonance energy transfer (FRET), diagrammatically represented in fig. 1. A fluorescent molecule absorbs photons with a characteristic wavelength and liberates again the thus absorbed energy within a very short time by the emission of photons, which gives rise to a measurable fluorescence. Since during this process there is a certain energy loss by heat evolution, the emitted photon has a characteristic, reduced energy content and therefore a modified wavelength compared with the previously absorbed photon (Stokes shift). Both parameters, namely the absorption wavelength and the Stokes shift (and the emission wavelength dependent thereon) are characteristic parameters of any fluorescent molecule and are a function of the characteristics thereof. A fluorescence-resonance energy transfer can be measured if two fluorescent molecules interact in a mixture with one another and the absorption wavelength of one overlaps with the emission wavelength of the other. In this connection

Amended sheet

enzymatic ligation and cloned in an expression vector using standard methods. It is also possible to use here a randomly complex population of baiting and catching proteins in order to obtain a library of possible interaction partners. According to the invention genetic information and coding DNA sequence means cDNA or genomic DNA, preferably cDNA.

Known, conventional vectors can be used as expression vectors. However, they should permit a separate selection of "baiting protein vectors" and "catching protein vectors" in the host cells, e.g. by different antibiotic resistance genes (e.g. ampicillin, kanamycin, chloroamphenicol, streptomycin, tetracyclines, sulphonamides) or different auxotrophic markers (e.g. LEU2, HIS3 or TRP1). However, it is also possible to combine both fusion proteins together in a bicistronic expression vector. Suitable expression vectors for the expression in E.coli are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b, pQE8 or pQE42, for the expression in yeast pY100, Ycpad1, pGBT9 or pGAD424, for the expression in animal cells pKCR, pEFBOS, cDMB and pCEV4. In particular, the Baculovirus expression vector pAcSGNt-A is suitable for expression in insect cells.

The expert is also aware of methods and host cells for expressing the expression vector and the fusion protein coded by it. Examples of such cells are the E.coli strains HB101, DH1, x1776, JM101, Jm109, B121 and SG13009, the yeast strain Saccharomyces cerevisiae, Schizosaccharomyces pombe, Y 190, CG1945, EGY48 or HF7, the animal cells L, 3T3, FM3A, CHO, COS, Vero and Hela and the insect cells Sf9. Suitable cells, preferably yeast cells, are (sequentially or synchronously) transformed with the expression vector or vectors and preferably propagated under double selection.

The fluorescence of the cells is then excited at the absorption maximum of one of the two fluorescent proteins (preferably at the maximum of FP-A), measured at the emission maximum of the other fluorescent protein (preferably of FP-B) and the measured result is used as a selection criterion in the isolation and cloning of cells containing a potential interaction pair. This measurement is calibrated beforehand by cells in which FP-A and FP-B are expressed cloned in different vectors, but without fusing with a further protein domain (negative control). The positive control can be constituted

by a fusion protein in which FP-A and FP-B (without baiting or catching protein) are coupled to one another in the above-described manner and consequently there is a maximum spatial proximity between FP-A and FP-B.

Fluorescence is preferably excited by laser. FRET measurement takes place using suitable filter combinations, which can be chosen by the expert as a function of the absorption and emission maxima of the fluorescent proteins used. In accordance with the bases of FRET each interaction between the fusion proteins must lead to a FRET rise, which can be detected after appropriate calibration of the system. For the investigation of known bait/catch pairs this detection preferably takes place by fluorescence microscopy. For the screening of cDNA libraries or combinatory peptide libraries measurement preferably takes place by fluorescence-controlled cell sorting (FACS).

For further identification of the interaction partners determined by FRET they are plated out on agar plates and plasmide-DNA is isolated from the individual colonies and can then be sequenced using standard methods.

The method according to the invention has e.g. the following preferred applications:

- A known pair of possible interaction partners is jointly expressed in a host cell and the resulting fluorescence spectrum is compared with the negative and positive controls or other interaction partners.

- A baiting-fusion protein is transformed or transfected with a randomly complex mixture of catching-fusion proteins (jointly or sequentially) in suitable host cells and preferably in such a way that each cell only carries one defined pair of possible interaction partners or a small group of such pairs. From the resulting host cell populations are then isolated the cells which have a significant FRET and consequently express a potential interaction pair. By isolating the expression vectors contained in these cells it is easily possible to determine the sequence of the participating interaction partners. The mixture of catching proteins could e.g. be made of cDNA libraries or combinatory peptide libraries.

The baiting protein could also be represented by a population of different proteins. It is consequently e.g. possible to determine an interaction matrix, e.g. in that a cDNA library is searched for possible interaction pairs between unknown proteins.

- A known pair of interacting fusion proteins is jointly expressed in host cells and the fluorescence transfer between the two fusion proteins is determined. Then a third protein or peptide is expressed in the same host cells and its influence on FRET is made an analysis criterion. This makes it possible to determine proteins or peptides which disturb or intensify an existing interaction between known interaction partners.

The advantages of the method according to the invention are that unlike in the "Yeast Two-Hybrid System" it is independent of transcription-regulating mechanisms and the location of fusion proteins in the nucleus is unimportant. Thus, e.g. also intact membrane proteins can be used as baiting proteins.

The possibility of an automated cell sorting additionally exists, which permits the obtaining of a high screening complexity. Through the combination of a pharmacologically interesting target sequence and a combinatory peptide library it is possible to screen several million peptide ligands per time unit. Compared with known methods with which this task might not be performable, the method according to the invention is characterized by an enormous time and labour saving.

The kit according to the invention (test kit) is described in claim 8. Preferred embodiments of this kit can be gathered from the dependent claims 9 to 16. The wording of claims 8 to 16 is hereby made by reference into part of the content of this description. Express reference is made to previous parts of the description directly or indirectly linked with the features of claims 8 to 16.

The claimed kit is used for performing the claimed method in a particularly simple manner. The kit preferably contains in a suitable container a first vector, which carries the host cell-optimized coding sequence of a FRET donor (under the control of a suitable promoter) and in which there is a cloning

possibility for the introduction of a coding sequence, which codes for a first test protein/test peptide (i.e. a baiting or catching protein or peptide).

There is also an associated second vector with host cell-optimized coding sequence for the associated FRET acceptor and cloning possibility for the coding sequence of a second test protein/test peptide (corresponding interaction partner for the bait-catch pair to be formed).

The coding sequences for the test proteins/test peptides in the first and/or second vector may already be present. Preferably also integrated cDNA libraries or combinatory oligonucleotide libraries are contained. In such cases e.g. a cDNA library (e.g. from the human brain) is present with one of the FRET partners fused in a first vector and a second vector in which the user can clone an own protein/peptide, e.g. as bait, with the other FRET partner.

The invention is described hereinafter relative to the drawings, wherein show:

Fig. 1 A diagrammatic representation of fluorescence-resonance energy transfer (FRET).

Fig. 2 A diagrammatic representation of the energy shift.

Fig. 3 A diagrammatic representation of FRET by means of the interaction of baiting protein with catching protein.

Fig. 4 A gene map of the expression vector pGBT9.

Fig. 5 The gene map of the expression vector pGAD424.

The invention is described by means of the following examples.

EXAMPLE 1

Initially a suitable vector system is formed. For this purpose and for

reasons of simplicity use is made of the plasmides pGBT9 (#K-1605-A), pGAD424 (#K1605-B) of Clontech (Palo Alto, US) and the plasmides pRSET B-P4-3 and pRSET B-S65T (Heim and Tsien, Current Biology 1996, 6: 178-182).

pGBT9 is a possible "baiting protein vector" of the Yeast Two-Hybrid System marketed by Clontech. The vector contains an ampicillin resistance gene for selection in bacteria, a TRP1 gene for auxotrophic selection in yeast and an expression cassette for the DNA-binding domain of the GAL4 transcription factor (fig. 4, Clontech Matchmaker "GAL4 Two-Hybrid Vectors Handbook, #PT3062-1) under the control of an alcohol dehydrogenase promoter (ADH1) for expression in yeast. The GAL4-DNA-binding domain is an essential component of the Yeast Two-Hybrid System.

For the purpose of the invention the GAL4 DNA-binding domain together with its nucleus location signal is removed, in that it is cut out by means of a HinDIII/EcoRI restriction digestion.

pGAD424 is a possible "catching protein vector" of the Yeast Two-Hybrid System (fig. 5). This vector has a similar structure to pGBT9, except that instead of the TRP1 marker it contains a LEU2 gene for selection in yeast and in place of the GAL4 DNA-binding domain it contains the GAL4 activating domain.

For the purpose of the invention also from this vector is removed by a HinDIII/EcoRI digestion the GAL4 activating domain. For this purpose initially further HinDIII restriction sites present in the vector are removed by partial digestion, filling the restricting site with Pfu polymerase and subsequent "blunt end ligation".

Suitable coding sequences of GFP variants S65T and P4-3 (Heim and Tsien, Current Biology 6: 178-182, 1996) are now amplified by PCR and provided at their 5' and 3' ends with HinDIII or EcoRI restriction sites. For this purpose use is made of the oligonucleotide primers GFP-Eco RI-3'(5'-CGGGAATTCTTTGTATAGTTCATCCAT-3'), and GFP-HinDIII-5'-TCCAAGCTTATGAGTAAAGGAGAAGAACTT-3') (Carl Roth GmbH, Karlsruhe). As the coding sequences of P4-3 and S65T are identical in their 5' and 3'-terminal

e.g. in that a cDNA library is searched for possible interaction pairs between unknown proteins.

- A known pair of interacting fusion proteins is jointly expressed in host cells and the fluorescence transfer between the two fusion proteins is determined. Then a third protein or peptide is expressed in the same host cells and its influence on FRET is made an analysis criterion. This makes it possible to determine proteins or peptides which disturb or intensify an existing interaction between known interaction partners.

The advantages of the method according to the invention are that unlike in the "Yeast Two-Hybrid System" it is independent of transcription-regulating mechanisms and the location of fusion proteins in the nucleus is unimportant. Thus, e.g. also intact membrane proteins can be used as baiting proteins.

The possibility of an automated cell sorting additionally exists, which permits the obtaining of a high screening complexity. Through the combination of a pharmacologically interesting target sequence and a combinatory peptide library it is possible to screen several million peptide ligands per time unit. Compared with known methods with which this task might not be performable, the method according to the invention is characterized by an enormous time and labour saving.

The kit according to the invention (test kit) is described in claim 7. Preferred embodiments of this kit can be gathered from the dependent claims 8 to 15. The wording of claims 7 to 15 is hereby made by reference into part of the content of this description. Express reference is made to previous parts of the description directly or indirectly linked with the features of claims 7 to 15.

The claimed kit is used for performing the claimed method in a particularly simple manner. The kit preferably contains in a suitable container a first vector, which carries the host cell-optimized coding sequence of a FRET donor (under the control of a suitable promoter) and in which there is a cloning possibility for the introduction of a coding sequence, which codes

for a first test protein/test peptide (i.e. a baiting or catching protein or peptide).

There is also an associated second vector with host cell-optimized coding sequence for the associated FRET acceptor and cloning possibility for the coding sequence of a second test protein/test peptide (corresponding interaction partner for the bait-catch pair to be formed).

The coding sequences for the test proteins/test peptides in the first and/or second vector may already be present. Preferably also integrated cDNA libraries or combinatory oligonucleotide libraries are contained. In such cases e.g. a cDNA library (e.g. from the human brain) is present with one of the FRET partners fused in a first vector and a second vector in which the user can clone an own protein/peptide, e.g. as bait, with the other FRET partner.

The invention is described hereinafter relative to the drawings, wherein show:

cells, once with the baiting plasmide pGBT-BFP-P2X2 and a further time with the empty baiting plasmide vector pGBT-BFP. The fluorescence spectra of the two transformants are then compared in order to exclude those interaction candidates which interact directly with the blue fluorescent protein, but not with the baiting protein.

Candidates checked in this way are then plated out again on selective minimum medium plates and the sequence of the potentially interacting catching domain is determined by standard molecular biology methods.

EXAMPLE 2

Use is made of two vectors, the first being selected from the commercially available expression vectors for EBFP or ECFP, e.g. pEBFP-N1, pEBFP-N2, pEBFP-N3, pEBFP-C1, pEBFP-C2, pEBFP-C3, pECFP, pECFP-C1 (Clontech) and the second from the commercially available expression vectors for EGFP or EYFP, e.g. pEGFP-N1, pEGFP-N2, pEGFP-N3, pEGFP-C1, pEGFP-C2, pEGFP-C3, pEYFP-N1, pEYFP-N2, pEYFP-N3, pEYFP-C1, pEYFP-C2, pEYFP-C3.

In one of the two vectors a DNA sequence is cloned in the usual way and this codes a baiting protein, so that the baiting protein is read off as a fusion protein with the fluorescent protein. In the other vector a cDNA library or a combinatory oligonucleotide library is cloned, so that the second fluorescent protein is read off as a fusion protein with in each case a protein fragment or combinatory peptide. Both vectors together are then transfected in known manner in suitable mammalian cells, e.g. COS-7, NG-108, HIH/3T3, etc.

Following an adequate incubation time the cells are then analyzed by FRET microscopy (see Clegg, in "Fluorescence Imaging Spectroscopy and Microscopy", pp 179-236, John Wiley & Sons, 1996) or in the above-described manner are individualized by fluorescence-activated cell sorting (FACS). The cells in which FRET occurs are then used in the conventional manner, e.g. by cytoplasm isolation with a micropipette conventionally used in electrophysiology and subsequent RT-PCR used for determining the basic catch sequences.

CLAIMS

1. Method for identifying peptide or protein interaction partners, in which at least two peptides or proteins are coupled to different fluorescent components, in which the absorption and emission spectra of the fluorescent components overlap in such a way that fluorescence-resonance energy transfer is possible and the fluorescence components can be brought together by an interaction between the proteins or peptides in such a way that FRET occurs and is measured, characterized in that peptides or proteins and fluorescent components in the form of fusion peptides or proteins are present and their genetic information is introduced into host cells in an expression system and FRET is measured there.

2. Method according to claim 1, wherein the fluorescent components with overlapping emission and absorption spectra are blue fluorescent protein and green fluorescent protein from Aequorea victoria.

3. Method according to claim 1 or 2, wherein the host cells are yeast cells.

4. Method according to one of the claims 1 to 3, wherein the measurement of the fluorescence-resonance energy transfer takes place by means of fluorescence microscopy or fluorescence-controlled cell sorting (FACS).

5. Method according to one of the claims 1 to 4, wherein fluorescence excitation takes place by laser.

6. Method according to one of the claims 1 to 5, wherein the protein or peptide fraction of the fusion protein or peptide comes from a combinatory peptide library or is the expression product of a cDNA library.

7. Kit for identifying interactions between proteins or peptides, comprising

a) at least one first vector with
   - a coding sequence for a first fluorescent protein or peptide and
   - a cloning site by means of which it is possible to introduce a coding sequence for a first test protein or test peptide and

b) at least one second vector with
   - a coding sequence for a second fluorescent protein or peptide, whose

Amended sheet

absorption spectrum overlaps with the emission spectrum of the first fluorescent protein or peptide in such a way that FRET occurs and

- a cloning site by means of which one coding sequence for a second test protein or test peptide can be introduced.

8. Kit according to claim 7, characterized in that the coding sequences for the first and second fluorescent protein or peptide are sequences optimized for a specific host cell.

9. Kit according to claim 7 or 8, characterized in that the first vector and second vector comprise a promoter able to function in a specific host cell.

10. Kit according to one of the claims 7 to 9, characterized in that the first vector and/or the second vector has at least one marker gene, whose expression in the host cell permits a selection of the cells containing the particular vector.

11. Kit according to one of the claims 7 to 10, characterized in that the coding sequence for the first test protein/test peptide and/or the coding sequence for the second test protein/test peptide is already introduced at the particular cloning site.

12. Kit according to claim 11, characterized in that the coding sequence is a so-called nucleotide library, preferably a cDNA library.

13. Kit according to one of the claims 7 to 12, characterized in that the first vector and/or the second vector is a plasmide, the corresponding cloning site preferably being a restriction detection site.

14. Kit according to one of the claims 7 to 13, characterized in that it additionally comprises a quantity of a suitable host cell type, in which the first vector and/or the second vector can be expressed.

15. Kit according to claim 14, characterized in that the host cell is a yeast cell.

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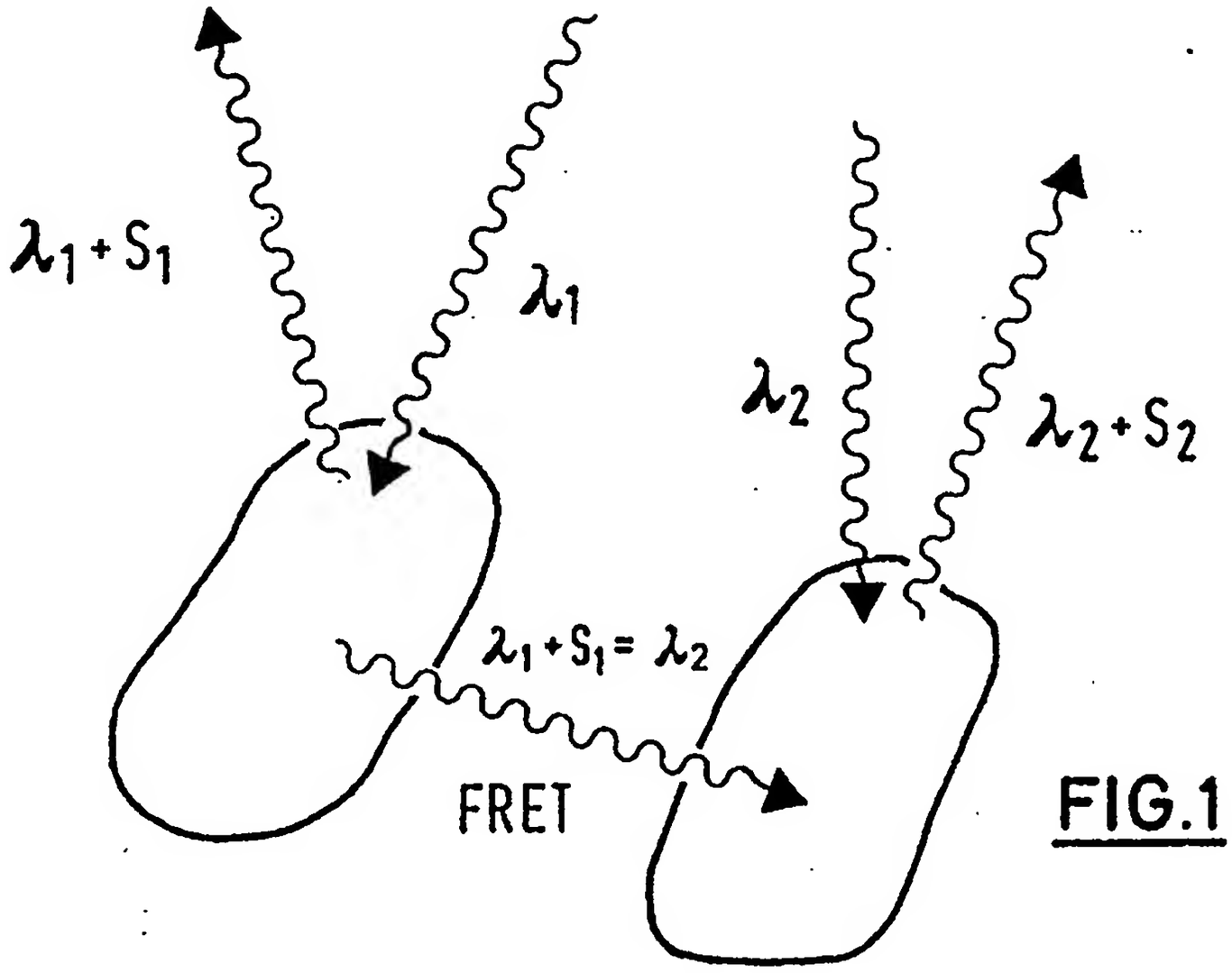

FIG.1

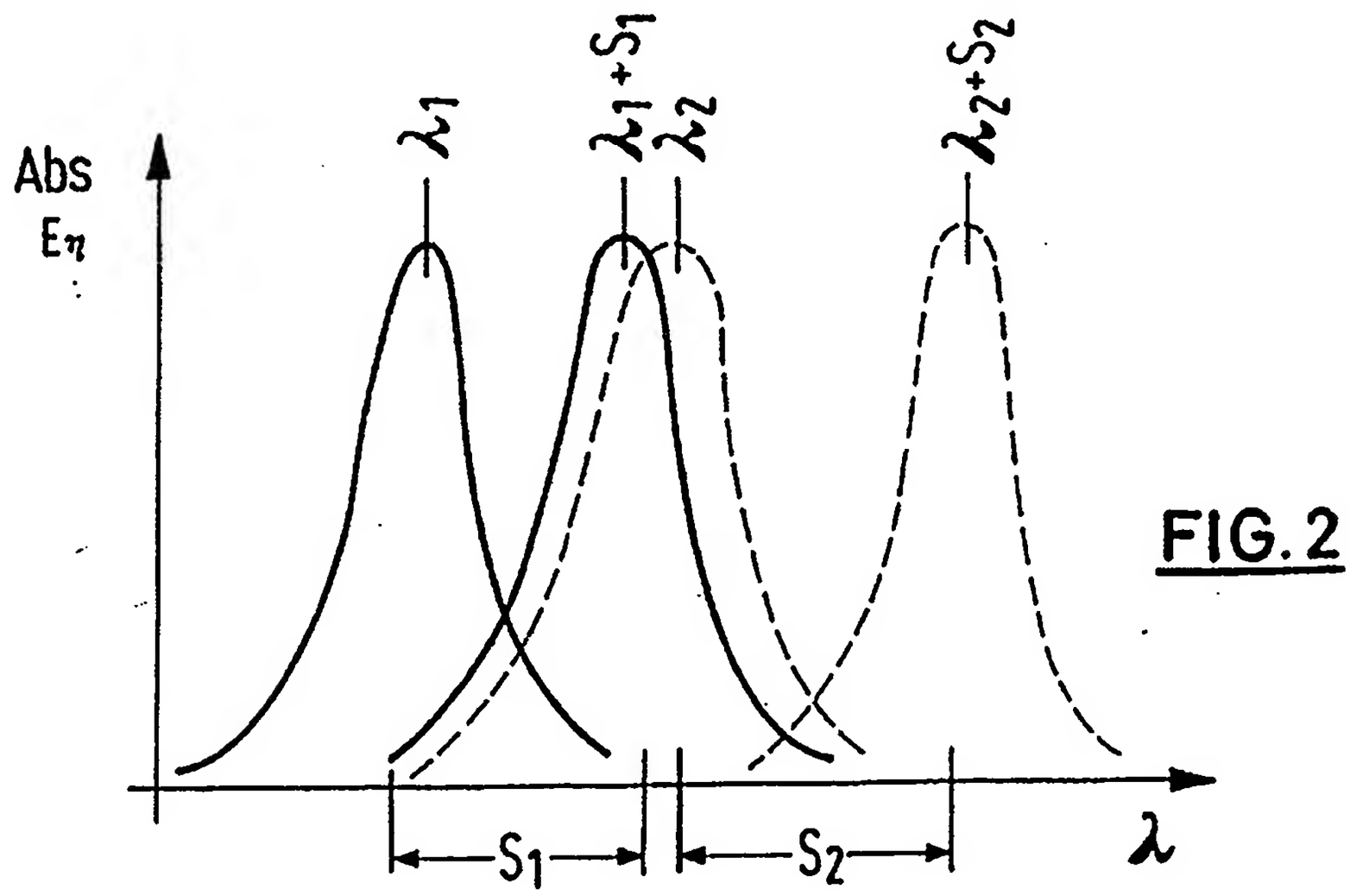

FIG.2

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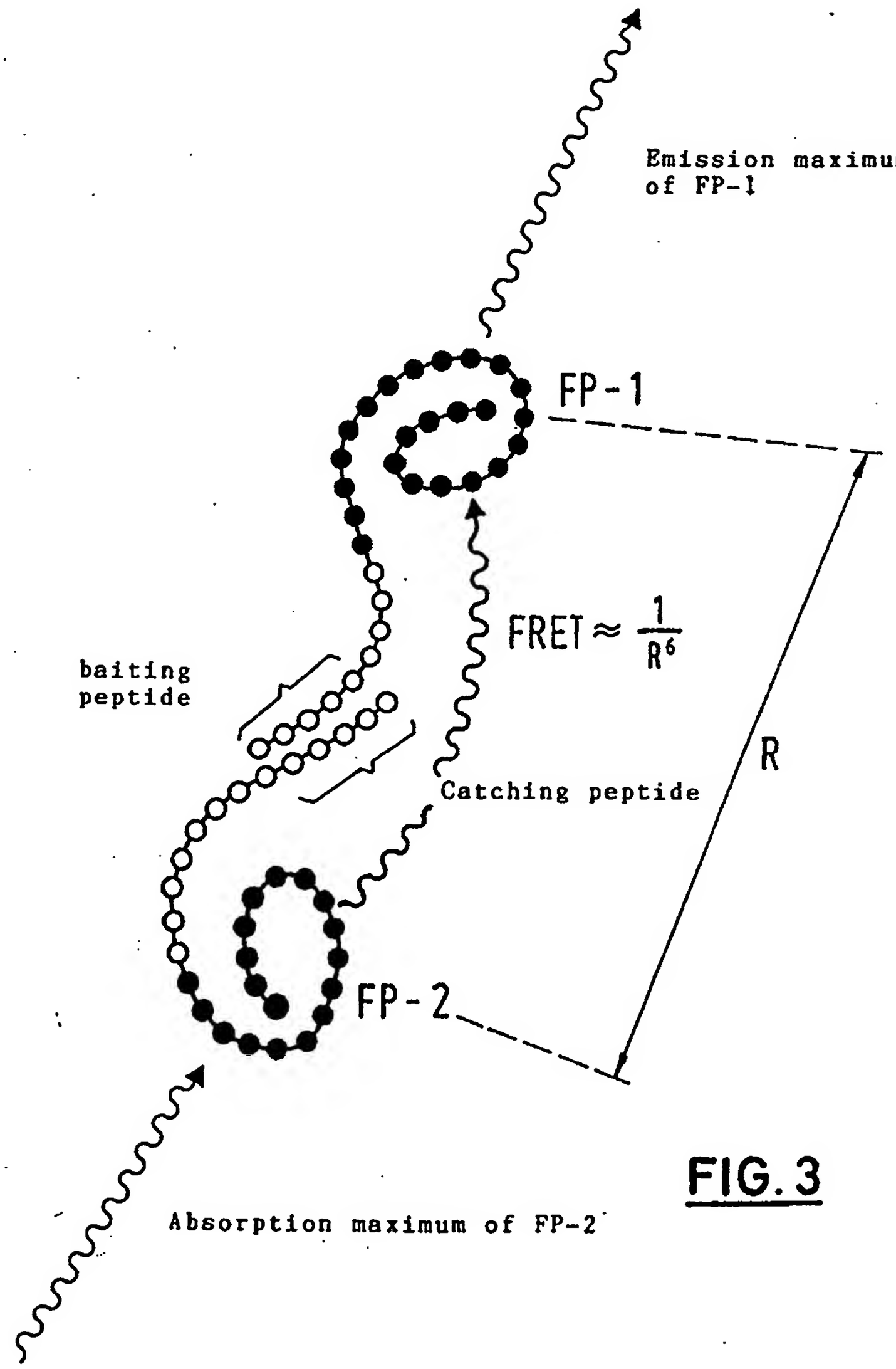

FIG. 3

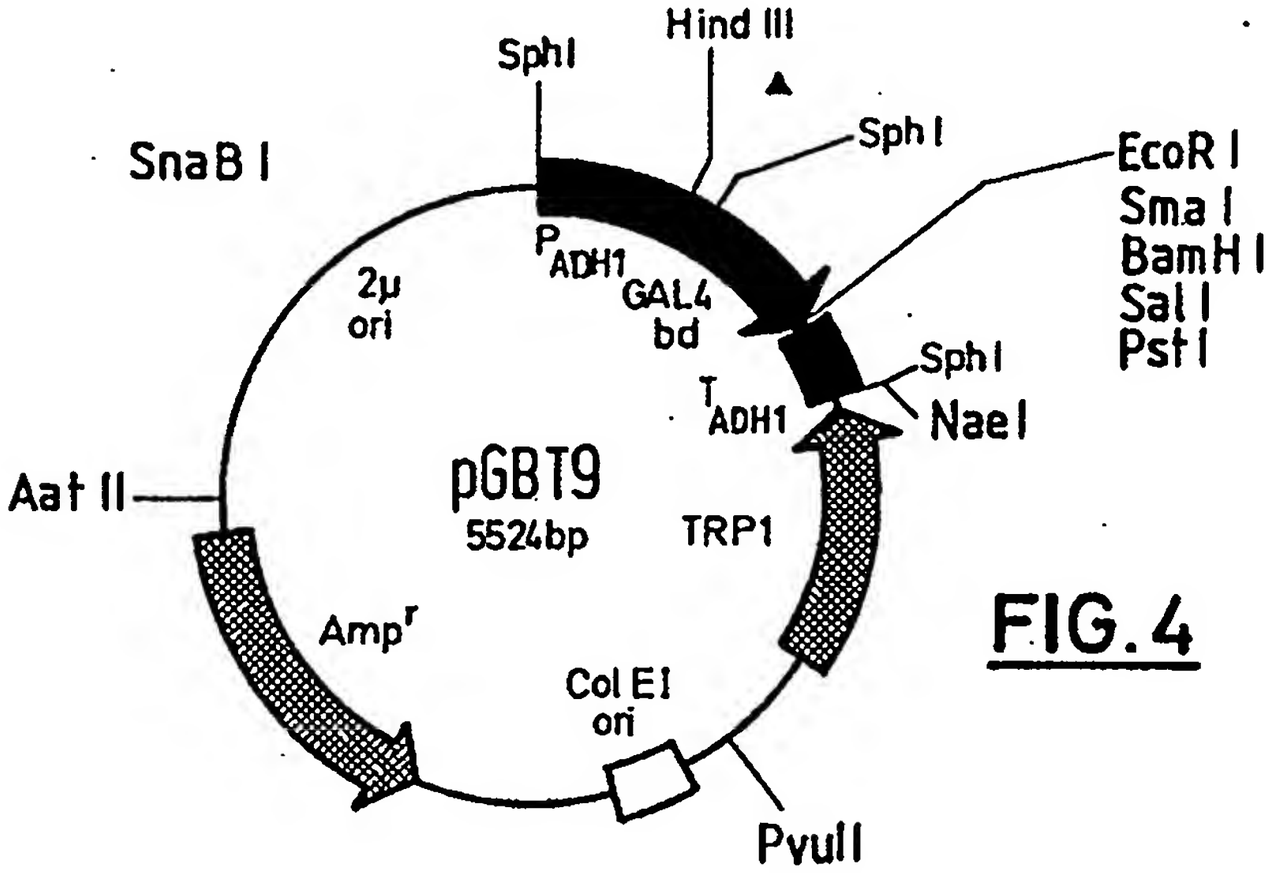

FIG. 4

T = Transcription termination sequence
▲ = SV 40 nucleus localization signal

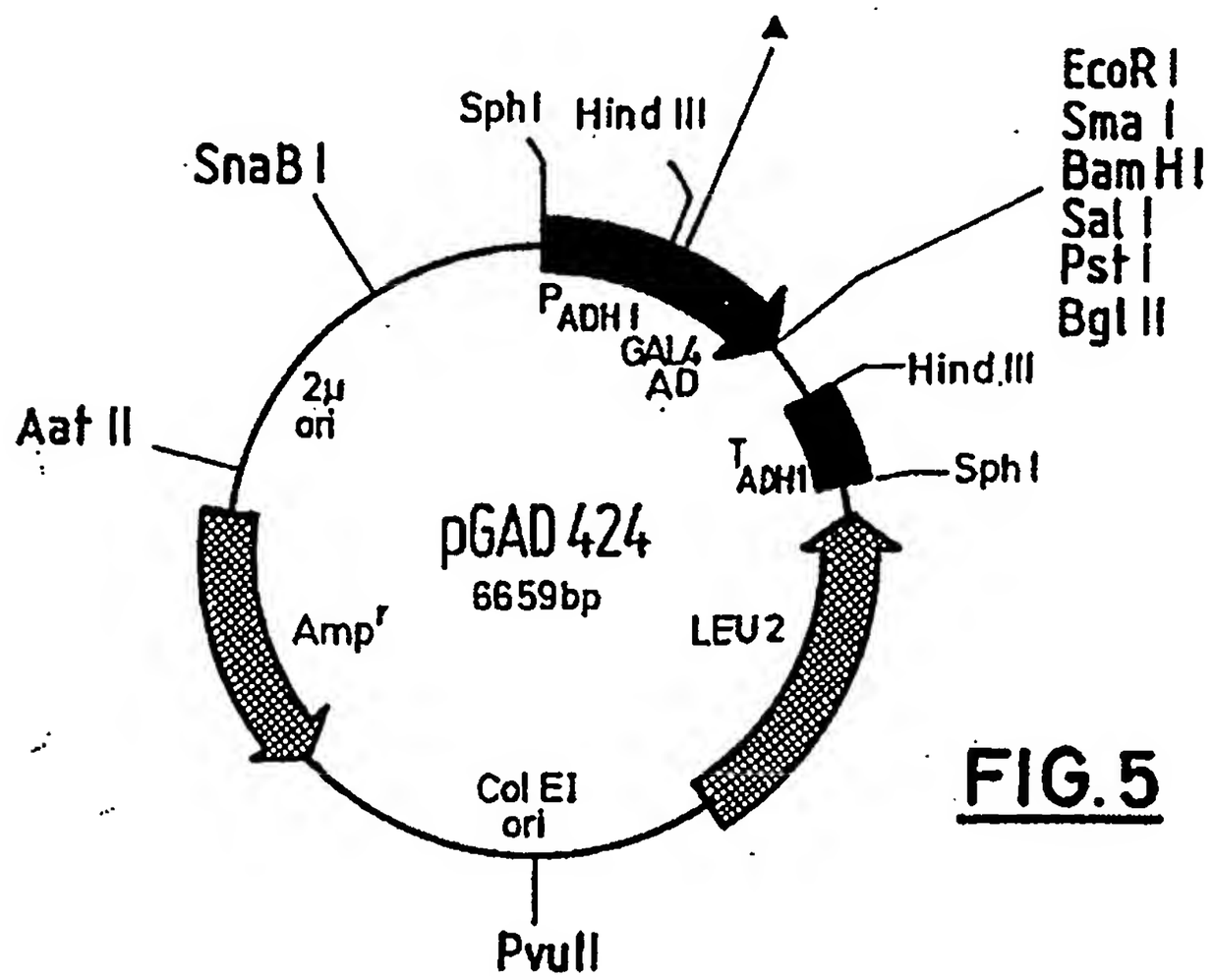

FIG. 5

T = Transcription termination sequence
▲ = SV 40 nucleus localization signal